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MOLECULAR METABOLISM

Genetic determination of the cellular basis of the ghrelin-dependent bone remodeling

Chengshan Ma [1], Toru Fukuda [2,3], Hiroki Ochi [2,3], Satoko Sunamura [2,3], Cheng Xu [1], Ren Xu [1], Atsushi Okawa [1], Shu Takeda [2,3,*]

ABSTRACT

Objective: Bone mass is maintained through a balance of bone formation and resorption. This homeostatic balance is regulated by various systems involving humoral and local factors. The discovery that the anorexigenic hormone leptin regulates bone mass via neuronal pathways revealed that neurons and neuropeptides are intimately involved in bone homeostasis. Ghrelin is a stomach-derived orexigenic hormone that counteracts leptin's action. However, the physiological role of ghrelin in bone homeostasis remains unknown. In this study, through the global knockout of ghrelin receptor (Ghsr) followed by tissue-specific re-expression, we addressed the molecular basis of the action of ghrelin in bone remodeling *in vivo*.

Methods: We performed molecular, genetic and cell biological analyses of Ghsr-null mice and Ghsr-null mice with tissue specific Ghsr restoration. Furthermore, we evaluated the molecular mechanism of ghrelin by molecular and cell-based assays.

Results: Ghsr-null mice showed a low bone mass phenotype with poor bone formation. Restoring the expression of Ghsr specifically in osteoblasts, and not in osteoclasts or the central nervous system, ameliorated bone abnormalities in Ghsr-null mice. Cell-based assays revealed ghrelin induced the phosphorylation of CREB and the expression of Runx2, which in turn accelerated osteoblast differentiation.

Conclusions: Our data show that ghrelin regulates bone remodeling through Ghsr in osteoblasts by modulating the CREB and Runx2 pathways.

Keywords Bone remodeling; Ghrelin; Osteoblast; CREB; RUNX2

1. INTRODUCTION

Bone remodeling, the function affected in osteoporosis, comprises two phases: bone formation by matrix-producing osteoblasts and bone resorption by osteoclasts [1,2]. The demonstration that the anorexigenic hormone leptin inhibits bone formation through a hypothalamic relay suggests that other molecule(s) affecting energy metabolism may also modulate bone mass [3–5]. Ghrelin is a peptide of 28 amino acids that was initially isolated from the rat stomach as an endogenous ligand of the growth hormone secretagogue receptor (Ghsr), which affects energy homeostasis by binding ghrelin [6]. Ghrelin has various physiological effects; it stimulates the appetite, increases food intake, and decreases energy metabolism through the Ghsr [7]. Clinically, gastrectomy is known to rapidly cause osteopenia, independent of nutritional defects such as insufficient calcium absorption [8], which indicates that the stomach regulates bone remodeling per se. Previously, we and others have demonstrated that ghrelin regulates osteoblast differentiation without affecting osteoclasts [9–11], and these findings also indicated that ghrelin may play an important role in bone remodeling *in vivo*. In contrast, a recent report using Ghsr-null mice showed that ghrelin regulates bone mass *in vivo* by regulating osteoclastic bone resorption through an unknown mechanism without affecting osteoblastic bone formation [12]. Thus, the physiological role of ghrelin in bone homeostasis remains to be determined.

In this study, through the global knock-down of Ghsr followed by tissue-specific re-expression, we addressed the molecular basis of the action of ghrelin in bone remodeling *in vivo*; these findings demonstrate that ghrelin regulates bone remodeling through osteoblastic Ghsr expression.

2. MATERIALS AND METHODS

2.1. Animals

Ghsr-null mice (Ghsr-null) [13], Osterix-Cre mice [14], Nestin-Cre mice [15] and Cathepsin K-Cre mice (Ctsk-Cre) [16] were described previously. The Ghsr-null mice were generated by inserting a loxP-flanked transcriptional blocking cassette (TBC) into a putative intron located downstream of the transcriptional start site and upstream of the translational start site of the murine *Ghsr* gene; this construct resulted in the knockout of *Ghsr* expression. Mating these Ghsr-null mice with tissue-specific Cre mice leads to the removal of the loxP-flanked TBC and enables the tissue-specific restoration of *Ghsr* expression in subsequent experiments [13]. We used littermates for all the experiments and we backcrossed at least eight times to ensure that mice are

[1]Department of Orthopedic Surgery and Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan [2]Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan [3]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Chiyoda-ku, Tokyo 102-0075, Japan

*Corresponding author. Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo Japan. E-mail: shu-tky@umin.ac.jp (S. Takeda).

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on a pure background. We bred Ghsr heterozygous mice with Ghsr heterozygous mice to obtain Ghsr-null and wild-type littermates. We also bred Ghsr-null mice with Ghsr-null/Nes-Cre or Ctsk-Cre or Osx-Cre mice to obtain Ghsr-null/Nes or Ctsk or Osx mice and Ghsr-null littermates. We maintained all the mice in a 12-h light–dark cycle with *ad libitum* access to regular food and water. All of the animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to the relevant guidelines and laws.

2.2. Cell culture

In vitro primary osteoblast and osteoclast-like cell cultures were established as previously described in Ref. [17]. Briefly, calvarial osteoblast cells were isolated from 4-day-old mice by enzymatic digestion in α-minimal essential medium (α-MEM) containing 0.5 mg/ml collagenase-P (Roche) and 0.05% trypsin. To induce osteoblast differentiation, primary osteoblasts or MC3T3-E1 osteoblastic cells were cultured in osteogenic medium (0.1 mg/ml ascorbic acid, 10 mM β-glycerophosphate) for 7 days after they reached confluence. The MEK inhibitor U0126, PKA inhibitor H89 and ghrelin were obtained from Promega, Calbiochem and the Peptide Institute, respectively.

In vitro osteoclast differentiation was carried out as previously described in Ref. [17]. Briefly, bone marrow cells from 6- to 8-week-old mouse femurs were cultured in α-MEM supplemented with FBS in the presence of human macrophage colony-stimulating factor (M-CSF, 10 ng/ml; R&D Systems) for 2 days and then differentiated into osteoclasts using human RANKL (50 ng/ml; PeproTech) and M-CSF for 3 days.

The osteoblast proliferation assays were performed using the Cell Counting Kit-8 (DOJINDO) according to the manufacturer's instructions.

All the results are representative of more than three individual experiments.

2.3. Quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen), and reverse transcription was performed with the ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. We performed quantitative analysis of gene expression using an Mx3000P real-time PCR system (Agilent Technologies). The mRNA levels are expressed relative to the housekeeping gene *Gapdh* and were calculated by the comparative threshold cycle (ΔΔCt) method.

2.4. Western blot analysis

Western blot analysis was performed according to a previously described standard protocol [17]. Primary antibodies against p38α, phospho-p38α, SAPK/JNK, phospho-SAPK/JNK, p44/42, and phospho-p44/42 (1:1,000; Cell Signaling); β-actin (1:2,000; Sigma); and Runx2 (1:1,000; Santa Cruz) were used.

2.5. Dual-luciferase reporter assay

We transfected MC3T3-E1 cells using Lipofectamine 2000 (Invitrogen). The activities of firefly luciferase and Renilla luciferase were determined using the dual-luciferase reporter assay (Promega).

2.6. *In vivo* studies

For intracerebroventricular (ICV) injection experiments, ghrelin (10 nmol/kgBW/day) was infused intracerebroventricularlly to 3-month-old female mice for 25 days as previously described in Ref. [5]. Briefly, a hole was made in the skull using a needle at a position 1.0 mm lateral to the central suture and 1.0 mm posterior to the bregma. A cannula was inserted into the third ventricle through the hole and connected to an osmotic pump (ALZET® Osmotic Pumps) placed in the dorsal subcutaneous space of the mouse.

All mice were housed individually in cages. The food intake of the mice was measured by subtracting the amount of uneaten food from the initial amount of premeasured food every morning at 10:00.

We also measured the visceral fat mass weight at 25 days.

2.7. Histological and histomorphometric analyses

We injected calcein (25 mg/kg, Sigma) i.p. 5 days and 2 days prior to sacrifice. We then stained the non-decalcified sections of the third and fourth lumbar vertebrae using the von Kossa technique. We performed static and dynamic histomorphometric analyses using the Osteomeasure Analysis System (Osteometrics). We analyzed 8–10 mice in each group.

2.8. Micro-CT analyses

We obtained two-dimensional images of the distal femur and femoral diaphysis by micro-CT analysis (Comscan). The following three dimensional morphometric parameters were determined using TRI/3D-BON software (RATOC): trabecular bone fraction (BV/TV), trabecular thickness (Tb. Th), trabecular number (Tb. N), trabecular separation (Tb. Sp), trabecular spacing (Tb. Spac), cortical volume (CtV), cortical thickness (CtTh), cortical perimeter (Ct. Perimeter), and bone mineral density (BMD).

2.9. Measurement of growth hormone (GH), insulin-like growth factor 1 (IGF1) and Tartrate-resistant acid phosphatase 5b (TRACP5b)

We measured the serum levels of GH, IGF1 and TRACP5b using a GH ELISA kit (Millipore), an IGF-1 ELISA kit (Alpco Diagnostics) and a TRACP5b ELISA kit (Immunodiagnostic Systems Ltd.) according to the manufacturers' instructions.

2.10. Statistical analysis

All data are represented as the mean ± s.d. ($n = 5$ or more), unless specified. We performed statistical analysis using Student's t-tests. Differences were considered to be statistically significant at $P < 0.05$. The results are representative of more than three individual experiments.

3. RESULTS

3.1. Ghsr signaling is required for proper bone formation and bone mass accrual

To address the role of ghrelin in bone remodeling, we first examined the expression of *ghrelin* (*Ghrl*) and its receptor (*Ghsr*) in bone tissue, osteoblasts and osteoclasts (Figure 1A). Ghrelin was expressed in bone cells at lower levels than in the stomach (Figure 1A). Substantial expression of *Ghsr* was observed in bone (Figure 1A), although the level of *Ghsr* in the bone was lower than that observed in the brain (Figure 1A), where *Ghsr* expression is known to be high [7]. Because ghrelin exerts its actions through the Ghsr, we next took advantage of Ghsr-null mice [13] to study whether ghrelin-Ghsr signaling regulates bone mass *in vivo*. Ghsr-null mice were created by inserting a loxP-flanked transcriptional blocking cassette (TBC) into a putative intron located upstream of the transcriptional start site of the *Ghsr* gene, which hampered Ghsr expression throughout the body.

Indeed, the expression of *Ghsr* was significantly decreased in the bones of Ghsr-null mice (Figure 1B). We analyzed both male and female Ghsr-null mice at 3 months and observed that male and female Ghsr-null mice exhibit low bone mass phenotypes as shown by a decrease in

